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Escherichia coli prlC Gene Encodes a Trypsin-Like Proteinase Regulating the Cell Cycle

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Proteinase In has previously been described as displaying a trypsin-like proteinase activity that momentarily appears immediately before DNA synthesis in the cell cycle of *Escherichia coli* synchronized by phosphate starvation and which is closely related to the initiation of DNA replication [Kato, M., Irisawa, T., Ohtani, M., and Muramatu, M. (1992) *Eur. J. Biochem.* 210, 1007-1014]. We purified the proteinase In from *E. coli* C600 and found that the 15 amino acid residues of its amino-terminal were identical with those of oligopeptidase A (OpdA), the product of the *E. coli prlC* gene. The purified proteinase had a molecular mass of approximately 67 kDa, which was also the same as that of oligopeptidase A. To further elucidate the relationship between proteinase In and oligopeptidase A, we assembled an expression vector to direct the synthesis of *E. coli* oligopeptidase A. The protein was expressed at a high level in *E. coli* BL21(DE3) and was produced mostly in the soluble, active form. Both the recombinant enzyme (rPrlC) and the purified proteinase In could hydrolyze trypsin substrates for proteinase In as well as benzyloxycarbonyl Ala-Ala-Leu *p*-nitroanilide (Z-AAL*p*NA), described as a synthetic substrate for oligopeptidase A. The effects of various protease inhibitors on rPrlC were also very similar to those on proteinase In. The trypsin inhibitors 4-guanidino benzoic acid 4-*tert*-butylphenyl ester and antipain strongly inhibited the trypsin-like proteinase activity of the recombinant enzyme, but had no effect on its Z-AAL*p*NA hydrolyzing activity. Cobalt ion, which greatly enhanced the OpdA activity, slightly inhibited the trypsin-like activity of the recombinant enzyme. These results strongly suggest that proteinase In is encoded by the *E. coli prlC* gene and is a multi-functional proteinase with two separate active sites.

Key words: cell cycle, *Escherichia coli*, gene expression, *prlC*, trypsin-like proteinase.

Recent advances in the study of cell cycle progression in eukaryotes have clarified the participation of cyclins and cyclin-dependent kinases (CDKs) in G1 phase progression (*1*), G1 phase to S phase transition (*2*), and G2 phase to M phase transition (*3*). Furthermore, we have reported that in HeLa cells synchronized by double-thymidine block, specific trypsin-like proteinases momentarily appeared in the late G1 phase (*4*), immediately before initiation of DNA synthesis (*5*) and in the late G2 phase (*6*), and that their inhibition by synthetic and specific trypsin inhibitors prolongs or blocks cell cycle progression.

In prokaryotes, such as *Escherichia coli*, no cyclins, CDKs, or other factors promoting cell cycle progression have been reported. Recently, Kato *et al.* reported the participation of a trypsin-like proteinase in the initiation of *E. coli* DNA synthesis (*7*), and named the purified enzyme proteinase In (*8*). Moreover, they reported that proteinase In is widely distributed not only in various *E. coli* strains (*9*) but also in *Bacillus subtilis* (*10*). Based on growth inhibition experiments with various synthetic trypsin inhibitors, they speculated that a proteinase In-like proteinase may occur in *Staphylococcus aureus* and *Staphylococcus epidermidis* (*11*). These findings strongly suggest the ubiquitous occurrence of proteinase In or a proteinase In-like proteinase in various microbes and its pivotal role in the onset of DNA synthesis. Although proteinase In has been well characterized (*8*), its amino-acid sequence has not been determined because it occurs in only minute amounts and requires a complicated purification procedure.

The *E. coli prlC* gene has been cloned and sequenced and shown to encode a 680-amino-acid protein (*12*). *E. coli prlC* and *Salmonella typhimurium opdA* are homologous genes (*12*). Oligopeptidase A (OpdA), the product of the *prlC* (*opdA*) gene, is an endoprotease which has been

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Abbreviations: The abbreviations used are: $AcAla_4$, *N*-acetyl-L-tetraalanine; Z-AAL*p*NA, benzyloxycarbonyl Ala-Ala-Leu *p*-nitroanilide; N-terminal, amino terminal; C-terminal, carboxyl terminal; Boc-Val-Pro-Arg-NH-Mec, 4-methylcoumaryl-7-amide of *tert*-butoxycarbonyl-L-valyl-L-prolyl-L-arginine; GBA-OPh^{t}Bu, 4-guanidino benzoic acid 4-*tert*-butylphenyl ester; Bz, N^2-benzoyl; Suc, succinyl; Glt, glytaryl; GBA, 4-guanidino benzoic acid; GMCHA, *trans*-4-guanidinomethylcyclohexanecarboxylic acid; APCA, amidinopiperidine-4-carboxylic acid; ACHCA, *trans*-4-amidinocyclohexanecarboxylic acid; OPh^{t}Bu, 4-*tert*-butylphenyl ester; OPhPh, biphenyl ester; IC_{50}, inhibitor concentration required for 50% inhibition.

purified from both *E. coli* and *S. typhimurium* (*13, 14*). Oligopeptidase A is the major soluble enzyme in *E. coli* which is able to hydrolyze the free lipoprotein signal peptide *in vitro* (*13*) and is required for the normal development of phage P22 (*12, 15*). *N*-Acetyl-L-tetraalanine ($AcAla_4$) and Z-AAL*p*NA are usually regarded as specific substrates for OpdA (*14*). Although alleles of *prlC* can weakly suppress certain *lamB* and *malE* mutations (*16-18*), it does not have an essential role in protein export (*18, 19*). The most important function of the *E. coli prlC* gene may still be unknown.

During the purification of proteinase In from *E. coli* C600 and the determination of its N-terminal sequence, we found that the N-terminal 15-amino-acid residues were identical with those of OpdA, the gene product of *E. coli prlC*. This paper reports the purification of proteinase In and the cloning and high-level expression of the *E. coli prlC* gene, and investigates the relationship between proteinase In and OpdA. The high-level expression of *E. coli prlC* will probably facilitate studies on the function of the gene and its relationship to cell cycle regulation.

EXPERIMENTAL PROCEDURES

Materials—*E. coli* C600 was kindly provided by Professor Zhang Linyuan of the Nanjing Research Institute for Military Medicine. Oligodeoxynucleotides were synthesized in the Nanjing University Oligonucleotide Synthesis Laboratory. Enzymes and reagents for gene manipulation and expression of rPrlC (OpdA) were purchased from New England Biolabs (Beverly, MA) and Boehringer Mannheim. Synthetic fluorogenic substrates and inhibitors for trypsin were from the Peptide Institute (Osaka). All other chemicals were obtained from Sigma.

Methods—Assay for proteinase In activity: The hydrolytic activity of proteinase In was assayed as previously described by Kato *et al.* (*8*). One unit was defined as the amount of activity that could hydrolyze 1 pmol of fluorogenic substrate in 1 min in 1-ml reaction mixture.

OpdA assay: The procedure of Conlin and Miller (*14*) was used with modification, as follows. Proteinase (30 μl) was mixed with 150 μl of a solution containing 50 mM Tris-HCl (pH 7.5) and 0.5 mM cobalt chloride. Then, 17 μl of Z-AAL*p*NA (0.5 mg/ml in dimethyl formamide) was added, and the mixture was incubated at 37°C for 1 h. To each reaction mixture was added 5 μl of aminopeptidase I (0.25 U/μl) and incubation was continued at 37°C for 10 min. To enhance the detection of free *p*-nitroaniline, the reaction product was diazotized and coupled to *N*-(1-naphthyl)-ethylenediamine to produce an intense purple color (*20*).

Protein assay: The protein concentration was determined by the method of Lowry (*21*) using bovine serum albumin as the standard.

Purification of proteinase In: Unless otherwise stated, all purification procedures were carried out at 4°C using 0.1 M sodium borate, pH 8.0, containing 1 mM $CaCl_2$ as the standard buffer (buffer A). Glucose minimal medium (GMM) (*22*) was used for large-scale cultivation of *E. coli* cells. One liter of GMM contains 7 g K_2HPO_4, 3 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4$, 0.5 g sodium citrate, and 2 g glucose. Approximately 100 g *E. coli* C600 cells were harvested from 50 liters of the mid-log phase culture. The cells were resuspended in 1 liter of buffer A containing 0.15 M NaCl, frozen at −20°C, allowed to thaw, and then homogenized with a sonic dismembrator 550 (Fisher Scientific) in an ice-bath. The homogenate was cleared by centrifugation at 10,000×*g* for 30 min. The precipitates were washed twice with 500 ml buffer A and homogenized and centrifuged as described above. The supernatant fluids were collected and named the crude extract.

The crude extract was applied onto a DEAE-cellulose column (5.5×30 cm) equilibrated with buffer A containing 0.08 M NaCl and washed with 3 liters of the same buffer. The adsorbed proteins were eluted with 2.4 liters of a linear gradient of 0.08 M to 0.5 M NaCl in buffer A at a flow rate of 1.5 ml/min. Ten-milliliter fractions were collected and the active fractions were pooled and subjected to ammonium-sulfate fractionation followed by successive chromatographies on hydroxylapatite (5.5×14 cm), phenyl-Sepharose CL-4B (2.4×7 cm), Mono Q 10/10 (Pharmacia), Sephadex G-100 (3×93 cm), and L-arginine-Sepharose 4B (1.4×4 cm) columns. The chromatographic conditions of all the columns were almost the same as those described by Kato *et al.* (*8*) except for the Mono Q column chromatography, which was performed as follows. The eluate from the phenyl-Sepharose CL-4B column was concentrated with an Amicon YM30, dialyzed against 20 mM Tris-HCl buffer, pH 8.0, containing 1 mM $CaCl_2$, and applied onto a Mono Q 10/10 column equilibrated with the same buffer. The column was washed with 80 ml of the buffer and eluted with 200 ml of a linear gradient of 0 to 0.5 M NaCl in the buffer. Five-milliliter fractions were collected at a flow rate of 1 ml/min.

Protein sequencing: The amino-terminal sequence of the proteinase was determined by the method of Xu *et al.* (*23*) in the Biological Laboratories of Harvard University.

Cloning of E. coli prlC gene: Oligodeoxyribonucleotides with the sequences CCAGAATTCC ATG GCT ACT AAC CCG CTG CTG ACT CCC TTT GAA TTG and CTCGGATCCAAGC TTA GCC CTT AAT GCC GTA ATG were used to copy the *prlC* coding sequence (~2.1 kbp) from *E. coli* C600 genomic DNA (*12*). The product was purified by 0.8% agarose gel electrophoresis, cleaved with *Nco*I and *Bam*HI restriction endonucleases, and ligated into the vector pET11d (Novagen, Madison, WI). The ligation mixture was used to transform competent *E. coli* BL21(DE3) cells and plated on medium containing ampicillin. Bacterial colonies were selected, and 3-ml cultures were grown to allow screening of plasmids by restriction endonuclease digestion of the DNA. Plasmids containing an insert with the expected size were partially sequenced, and one containing the correct sequence was used for all subsequent studies. This plasmid is designated pET11d-*prlC*.

Expression and purification of the recombinant prlC gene product (rPrlC): E. coli cells harboring pET11d-*prlC* were grown in 10 ml of LB medium supplemented with ampicillin (50 μg/ml) at 37°C overnight and then used to inoculate 400 ml of fresh LB medium supplemented with ampicillin (25 μg/ml). The A_{600} was monitored, and when it had reached 0.6, isopropylthiogalactopyranoside was added to a final concentration of 0.5 mM. The cells were grown for an additional 2 h to allow expression of the recombinant protein and then harvested by centrifugation. The cells (~1.6 g) were resuspended in 30 ml buffer A containing 0.08 M NaCl and homogenized by sonication. The recom-

binant protein was found to be present mainly in the soluble fraction, and the expression level was estimated at as high as 69% of the total soluble proteins. The soluble fraction (31 ml) was applied onto a DEAE-cellulose column (2.4×10 cm) equilibrated with buffer A containing 0.08 M NaCl and washed with 250 ml of the same buffer. The adsorbed materials were eluted with 1.2 liters of a linear gradient of 0.08 M to 0.5 M NaCl in buffer A. Ten-milliliter fractions were collected at a flow rate of 1 ml/min, and the active fractions were combined (~73 ml).

Inhibition of proteinase activity: The assay for inhibition of proteinase activity was the same as the assay for proteinase activity described above except that the proteinase was premixed with various amounts of inhibitors. IC_{50} was defined as an inhibitor concentration for 50% inhibition of proteinase activity.

RESULTS

Purification and N-Terminal Sequencing of Proteinase In from E. coli C600—A summary of the purification of proteinase In is shown in Table I. The yield of enzyme activity was 17.2% with 6,292-fold purification. From analytical SDS-PAGE, only a single band with non-minor contaminants was detected by Coomassie brilliant blue R250 staining (Fig. 1). The molecular mass of the purified proteinase was approximately 67 kDa resulting from SDS-PAGE. Its isoelectric point was determined to be 4.9 by isoelectric focusing. The N-terminal 15 amino acid sequence was determined to be Thr-Asn-Pro-Leu-Leu-Thr-Pro-Phe-Glu-Leu-Pro-Pro-Phe-Ser-Lys. By searching the GenBank data base, the N-terminal sequence of proteinase In was found to be identical with that of oligopeptidase A (OpdA), *i.e.*, the gene product of *E. coli prlC* (*12*). The molecular mass of OpdA was reported to be approximately 68 kDa (*12*) which was almost the same as that of proteinase In.

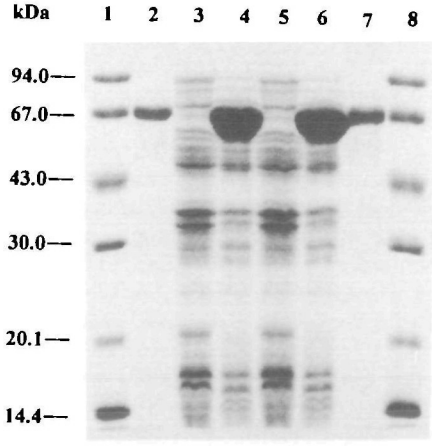

Fig. 1. **SDS-polyacrylamide gel electrophoresis.** Lanes 1 and 8, low molecular weight standards obtained from Pharmacia, with molecular masses indicated in kDa. Lane 2, approximately 4 μg of purified proteinase In stained with Coomassie brilliant blue R250. Lanes 3 and 4, whole cell lysates of *E. coli* containing the pET11d and pET11d-*prlC* plasmids respectively, after induction with isopropyl-thiogalactopyranoside. Lanes 5 and 6, the soluble fractions of Lanes 3 and 4, respectively. Lane 7, purified rPrlC (~5 μg). All samples were boiled in a sample buffer containing 50 mM 2-mercaptoethanol and analyzed by 12% SDS-PAGE.

Cloning and High-Level Expression of E. coli prlC Gene—The *E. coli prlC* gene was amplified by PCR from *E. coli* C600 chromosomal DNA and then cloned into the *Nco*I and *Bam*HI cloning site of pET11d to result in the recombinant expression vector pET11d-*prlC*. Transformants containing the plasmid pET11d-*prlC* were cultivated and assayed by SDS-PAGE to estimate the amount and size of the recombinant PrlC. rPrlC was detected on Coomassie brilliant blue R250 stained gel as a distinct band with a molecular mass of approximately 67 kDa, which was the same as predicted (*12*). rPrlC was produced mostly in the soluble fraction and amounted to approximately 69% of the total soluble proteins (Fig. 1). The specific activity of the total soluble proteins for Boc-Val-Pro-Arg-NH-Mec was 74,300 U/mg which accounted for approximately 64% of that of the purified proteinase In from *E. coli* C600 (116,400 U/mg). This percentage roughly coincided with the expression level.

After the one-step DEAE-cellulose column chromatography, the recombinant protein (rPrlC) was purified to near

TABLE II. **Comparison of hydrolytic activities of proteinase In and rPrlC on various fluorogenic substrates.** Hydrolytic activity was examined in buffer A containing 0.08 M NaCl and 20 μM of each substrate. The hydrolytic activity for Boc-Val-Pro-Arg-NH-Mec was defined as 100. n.d., not detected.

Substrate	Relative activity (%)	
	rPrlC (OpdA)	Proteinase In
Boc-Val-Pro-Arg-NH-Mec	100	100
Boc-Val-Leu-Lys-NH-Mec	61	60.8
Boc-Leu-Gly-Arg-NH-Mec	<0.1	<0.1
Boc-Phe-Ser-Arg-NH-Mec	<0.1	<0.1
Boc-Leu-Ser-Thr-Arg-NH-Mec	<0.1	<0.1
Bz-Arg-NH-Mec	19	15
Arg-NH-Mec	<0.1	<0.1
Boc-Glu-Lys-Lys-NH-Mec	<0.1	0.1
Boc-Ile-Glu-Gly-Arg-NH-Mec	<0.1	<0.1
Glu-Gly-Arg-NH-Mec	<0.1	<0.1
Suc-Ala-Pro-Ala-NH-Mec	<0.1	<0.1

TABLE I. **Purification of proteinase In from *E. coli* C600.** Proteinase In activity was examined with Boc-Val-Pro-Arg-NH-Mec as the substrate. n.d., not determined (because Tris-HCl buffer was used).

Purification step	Activity [units (U)]	Protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Crude extract	425,470	23,050	18.15	100	1
DEAE-cellulose	728,860	5,628	129.5	171.3	7
Ammonium sulfate	566,400	1,325	427.5	133.1	23.1
Hydroxylapatite	483,470	253.8	1,845.3	113.6	99.9
Phenyl-Sepharose	257,300	26.12	9,850	60.5	532.4
Mono Q	218,870	n.d.	/	51.4	/
Sephadex G-100	163,000	2.89	56,362.4	38.3	3,046.6
Arg-Sepharose	73,390	0.63	116,400	17.2	6,292

TABLE III. **Comparison of the inhibitory effects of various protease inhibitors on the trypsin-like activity of proteinase In and rPrlC.** Boc-Val-Pro-Arg-NH-Mec was used as the substrate of trypsin-like proteinase. IC_{50} was defined as the inhibitor concentration for 50% inhibition of proteinase activity.

Inhibitor	IC_{50} (μM)	
	rPrlC	Proteinase In
GBA-OPh'Bu	4	5
GBA-OPhPh	15	10
GMCHA-OPhPh	40	38
GMCHA-OPh'Bu	53	62
APCA-OPh'Bu	>200	>200
ACHCA-OPh'Bu	>200	>200
Leupeptin	14	10
Antipain	3.5	4.5
Chymostatin	5.5	6

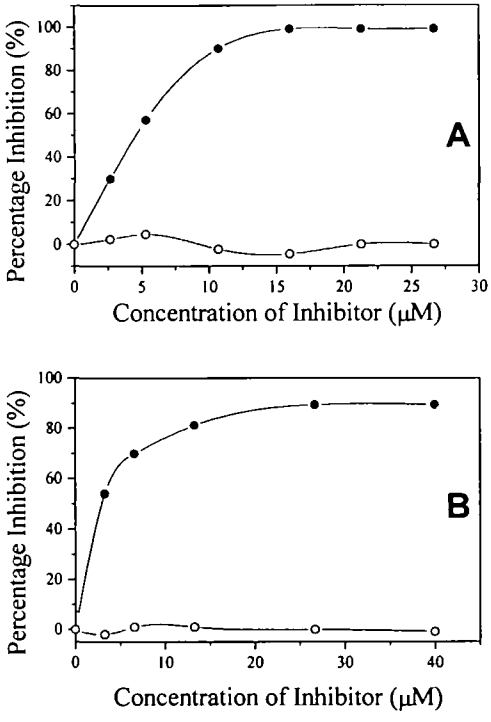

Fig. 2. **Inhibitory effects of GBA-OPh'Bu (A) and antipain (B) on rPrlC.** rPrlC was mixed with various amounts of the inhibitors and assayed for its Boc-Val-Pro-Arg-NH-Mec (●) and Z-AAL*p*NA (○) hydrolyzing activities.

homogeneity (Fig. 1). The purified rPrlC could clearly hydrolyze Boc-Val-Pro-Arg-NH-Mec, which was the specific substrate for proteinase In (see below), and had a specific activity of 107,000 U/mg for the substrate. The isoelectric point of rPrlC was determined to be 4.9, which was the same as that of proteinase In.

Substrate Specificity of Proteinase In and rPrlC—The hydrolytic activity of rPrlC on various fluorogenic substrates is shown in Table II and compared with that of the purified proteinase In. Both proteinase In and rPrlC clearly hydrolyzed trypsin-specific substrates, particularly Boc-Val-Pro-Arg-NH-Mec, and would appear to require a hydrophobic amino acid sequence lying towards the N-terminal from the P1 residue (lysine, arginine) for maximal activity. The K_ms of proteinase In and rPrlC for Boc-Val-Pro-Arg-NH-Mec were 63 and 71 μM, respectively. Be-

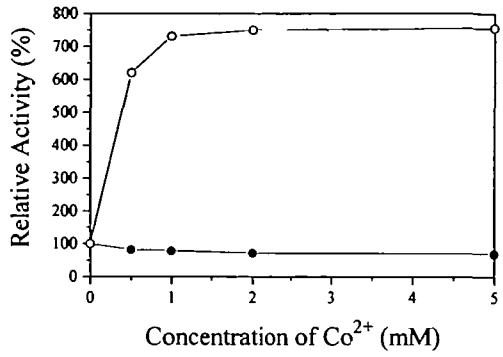

Fig. 3. **Effect of bivalent cobalt cation (Co^{2+}) on rPrlC.** rPrlC containing various concentrations of Co^{2+} was assayed for its Boc-Val-Pro-Arg-NH-Mec (●) and Z-AAL*p*NA (○) hydrolyzing activities.

sides trypsin-specific substrates, Z-AAL*p*NA was also hydrolyzed by proteinase In and rPrlC, for which their K_ms were 43 and 36 μM, respectively. Based on these data, we propose that proteinase In and rPrlC (OpdA) are actually the same proteinase.

Effects of Various Protease Inhibitors on the Trypsin-Like Activity of Proteinase In and rPrlC—Table III shows the inhibitory effects of various protease inhibitors on rPrlC and proteinase In. These data also indicated the identity of proteinase In and rPrlC (OpdA).

Figure 2 shows the effects of GBA-OPh'Bu and antipain on the Boc-Val-Pro-Arg-NH-Mec and Z-AAL*p*NA hydrolyzing activities of rPrlC. Both of the trypsin inhibitors strongly inhibited the Boc-Val-Pro-Arg-NH-Mec hydrolyzing activity of rPrlC, and they each had an IC_{50} of less than 5 μM. It was a little surprising to find that neither of the inhibitors inhibited the Z-AAL*p*NA hydrolyzing activity of rPrlC, from which we surmise that the two substrate-hydrolyzing active sites might be located in two different domains of rPrlC (OpdA).

Figure 3 shows the effect of the bivalent cobalt cation (Co^{2+}) on the Boc-Val-Pro-Arg-NH-Mec hydrolyzing activity of rPrlC. Co^{2+} slightly inhibited the Boc-Val-Pro-Arg-NH-Mec hydrolyzing activity of the proteinase while stimulating its oligopeptidase A activity markedly (Fig. 3) (*12, 13*). This result also suggests that OpdA is a proteinase with two separate active sites.

DISCUSSION

E. coli prlC contains an open reading frame of 680 amino acids, and the deduced molecular mass of the gene product should be 77 kDa (*12*), however, the experimental result was always approximately 67 kDa. We suppose that the difference was due to the posttranslational process. Since the N-terminal amino acid sequence of proteinase In (PrlC) determined in our study was identical with that deduced from the nucleotide sequence (*12*) and therefore intact, the posttranslational process of PrlC probably occurred at its C-terminus and most likely by autoprocess, because the molecular mass of rPrlC expressed at a high level was also approximately 67 kDa.

Two general approaches have been used to define genetically the genes that encode components of the cellular protein export machinery. One of these strategies identifies mutations that confer a conditional-lethal, pleiotropic

export defect (*sec*, *sec*retion). The other identifies dominant suppressors of signal sequence mutations (*prl*, *p*rotein *l*ocalization) (*24*). Although most of the products of *sec* and *prl* genes are membrane-binding proteins and are essential for the *E. coli* protein export machinery, some of these gene products, especially SecB and PrlC, are cytoplasmic proteins and do not play an essential role in the *E. coli* protein export (*19, 24*). Since biochemical studies demonstrate that SecB is an antifolding factor (*24*) and is presumably reused many times, it fits the definition of a molecular chaperone (*25*). Genetic analysis suggests that *prlC* does not specify an essential function in *E. coli* protein export and is therefore not a *sec* gene by definition (*24*). Furthermore, we found that during its expression and purification, rPrlC was very stable and highly soluble even at a high concentration (data not shown)—features that suggest PrlC may also be a molecular chaperone.

It is generally considered that the initiation of DNA replication (G1/S phase) is the key regulation point of the *E. coli* cell cycle and that DnaA protein is the key regulator determining the timing of initiation (*26*). Several hypotheses have been proposed to explain the mechanism by which DnaA protein might function, but none of them is perfect (*26*). Since *de novo* synthesis of DnaA is required for the reinitiation of *E. coli* DNA replication, it was long believed that a gradual increase in the DnaA concentration triggers the initiation of *E. coli* DNA synthesis. However, recent studies suggest that the principal regulatory factor is not the gradual accumulation of some product, or a gradual increase in the DnaA:*oriC* ratio, but rather the sudden appearance of a large excess of initiation potential, triggered by the synthesis of a particular regulatory factor (protein or metabolite) exclusively during a brief interval in the cell cycle (*27*). Proteinase In (PrlC) is a likely candidate for such a regulatory factor by virtue of the transient and periodical appearance of its trypsin-like activity in the cell cycle. The excellent work by Katayama and Nagata (*28*) suggests that in the wild-type *E. coli* cell, only newly synthesized, partially folded DnaA is active for initiation. If PrlC is a molecular chaperone, then it may act as a binding protein for newly synthesized DnaA to prevent it from folding completely and thus losing its initiating activity. We are now studying the relationship between DnaA and PrlC to confirm the hypothesis.

In the present paper, we have proved that proteinase In and *E. coli* PrlC (OpdA) are actually the same proteinase. In previous reports, on the one hand, the proteinase was described as the major soluble enzyme in *E. coli* capable of hydrolyzing the free signal peptide and as having substrate specificity for $AcAla_4$ or Z-AAL*p*NA (*13*), while on the other hand, it was reported to be a trypsin-like proteinase closely related to the synchronized *E. coli* cell cycle (*8*). Our experiment on the inhibitory effects of GBA-OPh^{t}Bu and antipain toward rPrlC clearly showed that both these specific inhibitors only inhibit the trypsin-like proteinase activity of rPrlC, and not its Z-AAL*p*NA hydrolyzing activity. In addition, cobalt ion greatly enhanced the Z-AAL*p*NA hydrolyzing activity of rPrlC but slightly inhibited its trypsin-like activity (Fig. 3). These results strongly indicate that proteinase In (PrlC) possesses two separate active sites localized in different domains. A comparison of the aligned protein sequence of PrlC with known sequences of proteases and oligopeptidases derived from the protein data bank did not reveal any sequence homology. Thus, the possible two active sites of PrlC will have to be analyzed on a structural basis by crystallography. Crystallization of the recombinant PrlC protein is now under way. The following hypothesis may explain our results. The signal peptide hydrolyzing activity of proteinase In (PrlC) runs through the *E. coli* cell cycle while its trypsin-like proteinase activity is inhibited by certain specifically endogenous protease inhibitors or competing substances except in a short moment immediately before the DNA replication. Further work will be necessary to elucidate the cell cycle regulating function of proteinase In and the mechanism by which it functions.

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